Reproduction potentiated in nematodes (*Caenorhabditis elegans*) and guppy fish (*Poecilia reticulata*) by adding a synthetic peptide to their aqueous environment

Running title: Reproductive peptide

Keywords:

Caenorhabditis elegans
Poecilia reticulata (guppy)
breeding
fertility
fecundity
peptide

Authors & affiliations:

Keith G. Davies[1*], Brian Zimmerman[2], Ed Dudley[3], Russell P. Newton[3] & John E. Hart[4]

[1]*School of Life and Medical Sciences, University of Hertfordshire, College Lane, Hatfield AL10 9AB, UK*
[2]*Aquarium, Zoological Society of London, Regent's Park, London NW1 4RY*
[3]*Biochemistry Group, Institute of Life Sciences, Medical School, Swansea University, Swansea SA2 8PP, UK*
[4]*Endocrine Pharmaceuticals, Wilderness End, Tadley Common Road, Tadley, Hampshire RG26 3TA, UK*

* Corresponding author, email: k.davies@herts.ac.uk

Grant support: None

ABSTRACT

Ambient exposure to a short synthetic peptide has enhanced fecundity (number of offspring) in invertebrates and vertebrates, ostensibly by disinhibiting reproduction. In separate experiments nematodes (*Caenorhabditis elegans*) and guppy fish *(Poecilia reticulata)* were exposed via their aqueous environment to a dissolved synthetic hexamer peptide, IEPVFT (EPL036), at a concentration of 1 μM. In the case of the worms peptide was added to their aqueous buffer daily throughout the experiment (14 days); in the guppies peptide administration was on the first 15 alternate days in a 50-week experiment. Fecundity rose by 79% among the worms. The number of descendants of the treated guppies was more than four times that of controls by Week 26 (103 vs 25, including 72 juveniles vs 6), with 15.4% more estimated biomass in the test tank in total (i.e. including founders). It was deduced that treated females bred earlier, at a smaller size, and had larger brood sizes. The total number of fish in the control tank had caught up by termination, but biomass continued to lag the test tank. There were no overt signs of toxicity among either the worms or the fish. Bioinformatics has been unilluminating in explaining these results in terms of mimicry of an endogenous regulator. A mass spectrometric campaign to identify a receptor, using murine brain for expediency, proved inconclusive. Molecular modelling *in silico* indicated unexpectedly that the 6mer EPL036 might be acting as an antagonist, to profecundity effect; that is, as a blocker of an inhibitor. This suggests that there awaits discovery an evolutionarily conserved reproductive inhibitor and its (antifecundity) receptor.

INTRODUCTION

It has proved possible to enhance fecundity (number of offspring) of *Caenorhabditis elegans* by over 40%, with treated worms also living a fifth longer on average than untreated controls (Davies and Hart, 2008). This was brought about simply by administering a 14mer peptide, EPL001 (see Table 1 for amino acid sequence), to the aqueous medium in which this nematode was maintained, to achieve a concentration of 1 μM. The route of ingress – intestinal, cuticular – was not established and the physiological mode of action remained obscure. A localisation study did however show that this profecundity peptide, with a 5mer C-terminal linker for fluorescence labelling, accumulated in the genital tract of these hermaphrodite worms.

The peptide EPL001 arose from a hormone discovery programme directed at finding an endogenous tissue-mass inhibitor in mammals (Haylor *et al*, 2009, Hart, 2014). An N-terminal sequence of 14 amino acids was obtained from ovine material, but bioinformatics failed to identify the parent molecule. A 14mer peptide corresponding to this N-terminal sequence was synthesized. It was designated EPL001 and shown to have biological activity: inhibition of compensatory renal growth was observed in the rat following unilateral nephrectomy (Haylor *et al*., 2009). Since this system is associated with IGF1 and the related DAF2 pathway is known to be important in longevity and reproduction in *C. elegans* (Baumeister *et al*., 2006), it was of interest to test EPL001 in this nematode in terms of lifespan and fecundity (Davies and Hart, 2008). That both these factors were altered by the peptide was at least nominally consistent with the renal data and with the notion that the 14mer EPL001 might be related in some way to an endogenous factor.

Two other peptides, EPL030 and EPL040, were used as scrambled-sequence comparators for the profecundity EPL001 in the worm experiment (Davies and Hart, 2008). These peptides

reduced fecundity by more than 60%. Given these findings the present study, apart from taking the research into a vertebrate (guppy fish), involved an attempt to identify any receptors to which the 14mers EPL001 and EPL030 and the 19mer EPL040 might bind, using murine brain and a biotin-streptavidin system (Rife *et al*., 2003), followed by LC-MSMS. Immunohistochemistry using a human tissue array and an antibody raised against EPL001 had shown epitopes in renal cells but also in brain tissue. This was in line with the postulation of an undiscovered somatic inhibitor expressed in bodily tissues and centrally (Hart, 2014). Brain from the mouse was favoured for analysis additionally (i) on the assumption that lifespan, fecundity and tissue-mass controls probably involve evolutionarily conserved systems across the phyla, (ii) because of the greater tissue availability of mammalian brain, appropriate for a pilot experiment, as against the exiguous tissues of nematodes and fish, (iii) because brain is a soft, highly defined tissue requiring minimal sample preparation, potentially leaving sufficient uncontaminated intact receptor material to permit detection and (iv) because of our previous experience and success in examining murine brain proteins by LC-MS (Ding *et al.*, 2008). The present study thus spanned three species to elucidate an intriguing ‘reproductive peptide’ effect.

RESULTS

Nematodes

Each of the three hexamers (Table 1) had a statistically significant profecundity effect on the nematodes, as compared with controls (Fig. 1). The increases in the cumulative number of offspring for EPL016, EPL037 and EPL036 were 50%, 71% and 79% respectively (ANOVA $p < 0.02$).

EPL036 (IEPVFT) forms part of the 19mer EPL040 (Table 1). Compared with the 79% pro-fecundity effect of the 6mer the 19mer caused a reduction in fecundity of 63% in an earlier study (Davies and Hart, 2008). The antifecundity effect of EPL040 was confirmed in a subsequent study of 10 worms subject to individual monitoring (data not shown). This approach potentially enabled biomass to be estimated on the basis of size measurements and actually permitted an appreciation of diversity in terms of a rare example of a male in this otherwise hermaphrodite species and death through endotokia matricida (lethal hatching of eggs inside the parent). But individual monitoring was less helpful in achieving statistical significance than when worms were evaluated in reasonable bulk (e.g. 4 replicates of 10 worms, as in the present study).

Referring to survivorship, there were no significant changes in average lifespan between the groups, but the time taken for 50 % of worms to die was significantly longer among those exposed to EPL036 (IEPVFT) than controls, and this was especially true of worms exposed to EPL037 (LQPAHV)(Table 2).

Nematode bioinformatics on EPL001, EPL030 and EPL040 yielded moderate hits to hypothetical proteins, without suggesting a research direction. Meanwhile amino acid sequence

comparisons of the antifecundity peptides EPL030 and EPL040 had indicated a shared motif of potential interest, IEPVFT (synthesized as EPL036). A BLASTP search using this hexamer sequence yielded 13 exact hits including one to a nematode protein. This was an uncharacterised WD40 repeat-containing protein from *Caenorhabditis briggsae*, accession number A8X7A7. The orthologue to A8X7A7 was identified in *C. elegans*, again using BLASTP searches, as a hypothetical protein with the accession number Q8WQB4. The equivalent motif to IEPVFT in *C. elegans* was LQPAHV (synthesized as EPL037, Table 1). A CLUSTAL alignment of proteins, including the zebrafish orthologue, is shown in Fig. 2.

Guppies

Tank set up

There was no discernable difference in the concentrations of either ammonia or nitrate in the two tanks in the biofilter pre-experiment. The peptide cannot therefore be said to have changed the rate at which beneficial bacteria had become established. After four weeks the pre-experiment was terminated, with no difference between the test and control tanks in terms of water chemistry (data not shown).

Fish study

There were no mortalities during the study and at no point did the fish exhibit any signs of distress or ill health or show any physical abnormalities. Normal behaviour was observed in both tanks, including feeding, courting, sparring and chasing. Water quality remained consistent throughout the trial and the 6mer EPL036 did not affect it in any discernable way (NH_4 both 0.03mg/L; NO_2 test 0.01mg/L, control 0mg/L; NO_3 both 5mg/L; pH test 7.2, control 7.4; temperature, both 23ºC; alkalinity, test 95, control 100). .

The number of descendants is shown in Table 3. Peptide exposure for a month had the effect on guppies of increasing numbers such that by Week 26 there were over four times as many non-founding fish in the test tank as in the control tank, a visible difference. At this time point in fact (Fig. 3) the recognisably male and female adult fish in the two tanks, subtracting the founders, were 31 tests and 19 controls, the former figure being 63% higher than the latter. The female adults in the test tank were significantly smaller (~20%) than controls and there was tendency (non-significant) for the males to be smaller too. As regards juveniles ('Fry' in Fig. 3), there were twelve times as many in the test tank as in the control one.

By the termination of the study at Week 50 control numbers had surpassed those in the test tank. Including the 20 founding adults, the control tank had 222 fish at termination, the test tank 197. Biomass tells a different story however. At Week 26 the total length of guppies in the control tank was 931 mm (n = 45, mean body length = 20.7 mm SE ± 0.8). The corresponding figure for the guppies in the test tank was 1,780 mm (n = 123, mean body length = 14.5 mm SE ± 0.5; +91% compared with controls), with 15.4% higher biomass. At termination no body length measurements were made but observation revealed that there were far more large fish in the test tank than in the control tank, indicating greater biomass in the former than in the latter. The structure of the populations at termination were very different: it was not just a case of the control group catching up.

Receptor

Two murine brain proteins were identified as interacting uniquely with the profecundity 14mer EPL001 (Table 4). These proteins were the alpha subunit of ATP synthase and growth-associated protein-43 (GAP-43, also known as neuromodulin). A further protein interacting with EPL001 was arginine kinase. This was also shown to interact with the antifecundity

EPL040, but not with the similarly antifecundity EPL030. As for EPL030 itself, interacting proteins were tentatively identified as a nuclear hormone receptor and a 14-3-3 family protein. The peptide X_{corr} was sufficient to identify all the peptide fragments except one. The exception was RLGLTEFEAVKQM, which was identified by a second peptide from the same protein, arginine kinase. Note, though, that all the candidate receptors fell short in terms of stringent peptide scoring, which optimally requires more than one confidently identified peptide fragment per protein.

Molecular modelling *in silico* showed that for the 14mer EPL030 the amino acid residues also present in its 6mer derivative EPL036, Valine 12, Threonine 14 and Phenylalanine 13, are superficial and offer potential binding sites to any receptor (Fig. 4). The molecular model of EPL040 (not shown), which displays similar antifecundity activity in *C elegans* to that of EPL030, has almost identical intramolecular distances relating to these three residues. In EPL036, which was profecundity in *C. elegans*, the loss of 8 N-terminal residues from EPL030 results in the intramolecular distance between phenylalanine and valine increasing and the orientation around proline altering. This would be consistent with the larger EPL030 molecule binding at a receptor as an agonist. EPL036, either because of the absence of some binding ligands or due to their altered orientation, only partially fulfils the binding requirements, leading to unproductive binding and its acting putatively as an antagonist.

DISCUSSION

This work has seen the identification of a short peptide motif associated with enhanced reproductive activity, having proline in a central position. A potent exemplification is the hexamer IEPVFT (EPL036), which is profecundity in both nematode worms and guppy fish.

The starting point for the present investigation was the outcome of an earlier study in *C. elegans* (Davies and Hart, 2008). A 14mer peptide EPL030 and a 19mer peptide EPL040, when administered separately via the worms' aqueous environment at a concentration of 1 μM, reduced fecundity to a near-identical degree (-64%). The 14mers were shortened in the current study to a common hexamer, IEPVFT (EPL036), and administered once more to *C. elegans*. The antifecundity activity of the parent peptides was transformed into a *profecundity* effect, of +79%. In the earlier study the 14mer EPL001 provoked an increase in nematode fecundity of +43%. When EPL001 was likewise shortened in the present work to a hexamer with a proline at position 3, i.e. MKPLTG (EPL016), the profecundity activity in *C. elegans* was more than maintained (+50%).

The 6mer EPL036 given to guppies via their tank water was associated with accelerated fecundation, such that by the half-year mark there were over four times as many descendants in the test tank as in the control tank and 15.4% greater biomass (including founders). That said, by termination there were more fish in total in the control tank than in the test tank. The structure of the populations were very different, however, with far more large fish in the test tank, indicating an enduringly higher biomass. This implies that had another cycle of reproduction been accommodated the number of test guppies would have exceeded once more the number of control fish.

It is intriguing that merely adding peptide to the aqueous environment of nematodes and fish should alter fecundity. To simplify interpretations via a single-species protocol, the nematodes were fed dead bacteria (to excess). The biofilter in the fish tanks could not be sterilised, but a prior experiment involving biofilters without fish present indicated that biofilter microorganisms seem to be unaffected by the 6mer EPL036. The 1 μM administration level is not especially high, with no nutritional effect of the peptide likely, but there could be scope for dose reduction, since a tenth of this dose of the 14mer EPL030 caused an antifecundity effect in the earlier study (Davies and Hart, 2008).

Biological activity presumably betokens internalisation, which raises the issue of route of ingress. Fluorescence studies in the earlier *C. elegans* study (Davies and Hart, 2008) revealed pharyngeal accumulation, possibly indicating gut uptake. Transit across the cuticle is not precluded but is unlikely based on work in another nematode, *Ascaris suum* (Sheehy *et al*, 2000). Permeability was demonstrated for monomeric to trimeric D-phenylalanine derivatives, with permeability rising with an increase in methylation. Permeability was not demonstrated for a 7mer in the same series which was unmethylated and less lypophilic and hence more in line with the 6mer EPL036. In the guppies in the present study no route of ingress evidence is available, yet passage across the gill membranes is an obvious possibility. As to tissue distribution, little is known beyond the apparent accumulation of EPL001, the 14mer fecundity enhancer, in nematode ovaries, as shown by fluorescent labelling of the peptide (Davies and Hart, 2008).

Some of the peptides under discussion have been investigated at a commercial facility growing nematodes for biocontrol in gardening and horticulture. The 14mer EPL001 increased total nematode numbers in the insect parasitic *Steinernema feltiae* by 33.6 % and in the slug parasitic

Phasmarhabditis hermaphrodita by 27.2 % (Jeremy Pearce and John Godliman, Becker Underwood Ltd, Worthing, UK, unpublished data). The comparable figures for the 14mer EPL030 were 31.7 % and 24.8 % respectively. That there was no difference in direction of effect between EPL001 and EPL030, as there is in *C. elegans*, means that had one of these entomopathogenic nematodes been selected for the core study then a reproductively active fecundity motif comprising a 'prolinaceous sextet' would not have been identified. The 6mer EPL036 (IEPVFT) itself was tried only in *Phasmarabditis hermaphrodita*. Time to peak production of the saleable infective larvae was reduced by a week, i.e. a quarter, with numbers at this time point up by 45% on controls. This is a notable result as the peptide was administered daily via the worms' aqueous growth medium, which resembled liquid brick dust.

Treatment of fish with proteinaceous agents to stimulate reproduction and increase fecundity is common practice in aquaculture, such as in the production of caviar from sturgeons (Lagler *et al.,* 1977). Implants and injections of pituitary material have been used in both freshwater and marine species for food production. In goldfish, *Carassius auratus,* analogues of LH-RH, injected or implanted as pellets and co-administered with a dopamine antagonist, caused almost nine out of ten females to ovulate (Sokolowska *et al*., 1984). Peptide hormone treatment with IGF-I in the short-finned eel, *Anguilla australis,* yielded an increase in oocyte diameter (Lokman *et al*., 2007). The majority of fish of interest in aquaculture are egg-laying species that produce vast numbers of eggs with each spawning. In contrast guppies are small livebearing fish with internal fertilisation and comparatively small broods. Their ability to store sperm allows multiple broods to be born from one mating. It is known that female guppies that engage in multiple matings exhibit an increased brood size and shorter gestation period (Evans and Magurran, 2000). This possibility is unlikely however in the present study since behavioural observations did not indicate an increased period of courtship in the study tank versus the

control. Courtship and other behaviour remained consistent in the two groups, including in the test tank immediately after the addition of the peptide. Fecundity enhancement via implant or injection in guppies is infeasible due to their small size, so in the present trial the peptide was simply added to the water. Direct contact or absorption by the guppies would have taken place either via the gill membranes, through the skin or via ingested water. Whatever the case, fecundity and biomass were enhanced by a simple ecosystem modulation.

Whereas the test nematodes were exposed to peptide throughout their lives, the test fish were only exposed during the first month – this is on the assumption that a short peptide is unlikely to have persisted long in tank water after the administration period. That means that only the 20 founder fish were exposed, as there will likely have been insufficient time for them to produce offspring before exposure ceased. By the half-year mark the adult female guppies in the test tank, a mixture of mothers and daughters but presumptively not granddaughters, were on average about a fifth smaller than adult female controls ($p<0.01$). A trade-off between reproduction and growth in guppies has long been recognised (Reznick, 1983). Peptide exposure seems to have slowed the growth of the founding mothers but anomalously also of their daughters, suggesting that some additional factor may latterly have been in play (e.g. unsuspected competition for food or tank constraints relating to carrying capacity). Juvenile numbers were twelvefold higher in the test tank after six months than among controls, so a general redirection from female growth to reproduction seems indicated. It can be deduced that test females reproduced at a smaller size than controls and that they produced more fry per mating, an increase in brood size not related to mating frequency (see above). Improvements in egg quality may have been involved, but direct evidence on this is wanting.

What of the male guppies? There was a non-significant trend towards lower body weights among test males (Fig. 3). If test females were bearing young at smaller size and hence younger age, then the males were presumably sexually active earlier than would normally be the case. In a separate intervention, adding the 6mer EPL036 to the food freshwater angelfish (*Pterophyllum scalare*) caused egg and fry production when males and females were exposed together, not when either sex was exposed alone (Granville Hammond, Devon Discus Ltd, Newton Abbot, UK, unpublished data). Fecundity built cumulatively, as peptide was readministered in five-day cycles in food after each spawning. Note that in this independent investigator's hands the both-sexes-at-once approach, via food, also induced a pair of hard-to-breed tiger stingrays (*Potamotrygon tigrina*) to produce a pair of pups, as well as causing a colony of reproductively quiescent Tiffany catfish (*Rineloricaria teffeana*) to start breeding again. The same both-sexes-together approach has also worked in a conservation setting with two species of rare Malagasy frogs, the splendid mantella *(Mantella pulchra*) and the brown mantella (*Mantella ebenaui*) (Mike Bungard, Paignton Zoo, Devon, UK, unpublished data). The breeding of the brown mantella is believed to be the first in a zoo anywhere in the world. The 6mer EPL036 was dissolved in water and misted onto the frogs in their rainforest vivaria, to achieve percutaneous absorption.

The serendipitous IEPVFT (EPL036) scored a bioinformatics hit to a *Caenorhabditis briggsae* gene (Fig. 2). The orthologous gene in *C. elegans* yielded LQPAHV (EPL037) as the corresponding hexamer. When EPL037 was administered to *C. elegans* it increased fecundity by +71%, nearly as much as the +79% achieved with EPL036. An apparent positive effect of both peptides on survivorship to the 50% die-off point hints that the bioinformatic hit was real and that the protein encoded by the gene might be processed into a secreted ligand, mimicked by LQPAHV and the other prolinaceous sextets. Yet the protein encoded by the *C. briggsae*

gene is a WD40 scaffold protein, an unpromising hit. It is difficult to propose any model within which LQPAHV or IEPVFT is both a functional ligand in its own right and ultimately derived from a WD40 repeat-containing protein. There appear to be no examples of secreted ligands containing the WD 40 motif.

The *C. briggsae* bioinformatics hit is probably fortuitous. When the homologous hexamer from *C. elegans* was synthesized it was to let nature do the combinatorial chemistry. This analysis leads away from a dubious gene hit towards a ligand motif, serendipitously discovered, with a proline residue as a common denominator.

The receptor hunt, exploring as it did proteomic interactions, yielded no coherent narrative (Table 4). GAP-43 is considered to be a downstream component of signal transduction systems rather than a ligand-recognizing receptor, yet it might be relevant; ATP synthase and arginine kinase are key players in cellular energetics, which might be germane to an energy-intensive activity such as reproduction; the nuclear receptor has been specifically implicated in egg laying in various organisms; while the 14-3-3 protein is suggested to have a function in the regulation of organismal lifespans. Further work is required.

Worms and guppies exposed to the 6mer EPL036 bred ‘earlier and more’. It has been conceptualised that sexual maturation occurs in mammals when a reproductive ‘brake’, operating centrally, is lifted (Witchel and Plant, 2013). This schema could be relevant as molecular modelling yielded the insight that the 14mer EPL030 and the 19mer EPL040, both *antifecundity* in *C. elegans*, were likely inhibiting reproduction by acting as agonists at an as yet undetermined receptor. In this view the *profecundity* 6mer EPL036 would be seen as an antagonist, i.e. as blocking an inhibitor. This suggests that there is waiting to be identified an

evolutionarily conserved endogenous reproductive inhibitor, as has been proposed (Hart, 2014), along with its ‘anti-fecundity receptor’.

Efficacy after ambient administration and activity in both an invertebrate and a vertebrate species make the ‘fecundity peptide’ IEPVFT (EPL036) and related proline-containing moieties worthy of further study, from both a fundamental perspective and in terms of applications in fertility and fecundity enhancement.

MATERIALS AND METHODS

Peptides

Peptides were synthesized by a commercial supplier (Peptide Protein Research Ltd, Fareham, UK). Amino acid sequences and proprietary designations are shown in Table 1. The peptide EPL030 is a scrambled version of the profecundity 14mer EPL001, i.e. it has the same amino acids in a different order. The 19mer EPL040 is a different anagrammatical version of EPL001, with the addition of a five amino acid linker, GSGSK, to facilitate C-terminal fluorescence labelling. Although nominally control molecules, both EPL030 and EPL040 showed unexpected antifecundity activity in *C. elegans* (Davies and Hart, 2008). It was noted that by chance the two control peptides shared the sequence commonalities KLK and IEPVFT. The latter was held likely to be of more interest, since bioinformatics revealed a hit for IEPVFT to the *Caenorhabditis briggsae* proteome, as described below. It was synthesized as EPL036. The corresponding hexamer from *C. elegans* was determined bioinformatically to be LQPAHV. This was synthesized as EPL037.

EPL036 and EPL037 are hexamers with proline at position three. Curiosity was piqued when it was noted that the N-terminus (MKPLTG) of the profecundity 14mer EPL001 also had a proline at position three. This 6mer was synthesized as EPL016.

Nematodes

Caenorhabditis elegans (N2) was grown in Petri dishes on Nematode Growth Medium (NGM) agar, i.e. 3 g NaCl, 2.5 g peptone and 17 g technical agar in 975 ml distilled H_2O, which was then autoclaved. To this was added 1 ml of $CaCl_2$ (1M), 1 ml $MgSO_4$ (1M) and 25 ml KH_2PO_4 (1M, pH6), all from filter-sterilised stock solutions. Finally, 1 ml of 5 mg/ml cholesterol in 95% ethanol was added to the medium (Sulston and Hodgkin, 1988).

Nematodes were routinely cultured by growing the bacterium *Escherichia coli* OP50 overnight in LB medium, on a shaker placed an incubator (37^{o}C). Petri dishes (9 cm) were seeded with the OP50 to create a bacterial lawn overnight. Nematodes were added to these Petri dishes, which were then maintained at 20^{o}C in an incubator (Sulston and Hodgkin, 1988).

Nematode eggs were placed on NGM plates and allowed to develop into larvae. Groups of 10 larvae that were clearly post L2 dauer decision stage (L3/L4) were used in these studies and placed in autoclaved staining blocks containing 200 µl sterilized M9 buffer (Davies and Hart, 2008). They were fed on heat killed bacteria rather than live bacteria to simplify interpretations. Each treatment group and an untreated control group consisted of 4 replicates of 10 worms that were placed randomly in a humid chamber and maintained at 20^{o}C in an incubator.

The adult worms were transferred to fresh medium each day to prevent adults becoming mixed with developing offspring and to allow the latter to be counted (Davies and Hart, 2008). The peptides were administered each day to the new aqueous medium (M9 buffer) in which the nematode were maintained, to achieve an initial concentration of 1 μM. This meant peptide did not accumulate in the medium over time, and that the concentration never exceeded 1 μM.

Mean data were calculated from the four groups in each treatment for fecundity and survivorship. Statistical analysis involved the calculation of standard errors of these means and analysis of variance (ANOVA) and *t-tests*, as appropriate.

Nematode bioinformatics involved using the amino acid sequences of the peptides in BLAST searches against standard databases at NCBI and Wormbase.

Guppies

Guppies, *Poecilia reticulata*, are livebearer fish native to the Caribbean and northern South America, in the family Poeciliidae, which also includes mollies, swordtails and mosquitofish (Nelson, 1994). The founder population of the guppies at ZSL London Zoo originated from a collection at the Pitch Lake in Trinidad in the mid-1990s. Peptide was simply added to the guppies' tank water.

Tank set up

A pre-experiment was conducted during the period when the fish tanks were being conditioned for the experiment proper. The issue was the number of species in the experimental system and the question of direct and indirect effects. In the nematode experiment reported here, the worms were fed dead bacteria rather than their normal diet of live bacteria to simplify the experiment down to a single live species. This was not practical for guppies, as a biofilter was required in the tank, for water purification purposes. The presence of an operational biofilter in each of the test and control fish tanks might have rendered interpretation problematic: would any observed changes be due to a direct effect on the fish or an indirect effect via the microbial population of the biofilter? Newly set up aquaria undergo a period of instability in the first several weeks with nitrogenous wastes building up in concentration until bacteria that utilise them become established in the tank (Moe, 1992). The filter in an aquarium provides a matrix for heterotrophic bacteria to colonise. These bacteria convert fish wastes from ammonia to nitrite and eventually to nitrate. Two identical aquaria were each filled with 30 L of aged tap water. A new air-driven biological sponge filter was added to each tank and ammonium chloride was administered at 1 mg per L per day to provide a food source of ammonia for the bacteria to establish themselves.

Fish study

For the purposes of the trial 100 juveniles were selected at random from the main guppy colony and isolated in a separate 30 L aquarium for maturation. Within two months sexual dimorphism was observed and the fish were divided into separate populations of males and females in order to prevent reproduction prior to the start of the study. Female guppies have the ability to store sperm packets in the folds of the oviduct, so a single mating can produce several broods over time (Moe, 1992). For this reason virgin status was necessary for the females to avoid starting the trial with fish that were already gravid.

Two glass aquaria of 30 L each were filled with aged tap water and one AquaZoo air-driven sponge filter to provide biological filtration. A small amount of Java moss, *Vesicularia dubyana,* was added to each tank to provide cover for the fish, notably the fry, which might otherwise be subject to cannibalism. After five weeks the fish were old enough for sexes to be distinguished in the maturation tank and ten males and ten females were transferred to a peptide treatment tank and a control (peptide-free) tank. The two tanks were placed 2m off the floor on a shelf to minimise disturbance during the study. The fish were fed on a mixed diet of newly hatched *Artemia salinas* nauplii, Aquarian brand vegetable flake food, frozen *Daphnia* sp. and *Cylops* sp.. Food was provided twice daily at 9am and 3pm. Fish were fed to satiety. The fish were observed daily for ten minutes during routine maintenance and feeding to assess behaviour.

The water quality parameters were tested weekly throughout the experiment using a Palintest photometer (Tyne & Wear, England) for pH, ammonia, nitrite and alkalinity. Temperature was

458 measured daily using a glass thermometer and nitrate was measured weekly using a Salifert test
459 kit (Duiven, The Netherlands).
460
461 Peptide EPL036 was added to the water of the test tank as an aqueous solution (<1ml) from pre-
462 dispensed vials of equal concentration. The first dose was administered on 14th January 2010,
463 during Week 1, and then doses were given every other day for a total of 15 doses (including
464 weekends). In the nematode study also reported here it was found expedient to move the
465 founding population of adults into fresh medium each day to facilitate counting and monitoring.
466 With daily peptide administration to the worms, this meant that dose levels never exceeded 1
467 μM. For the guppies, alternate-day dosing reduced the likelihood of peptide accumulating above
468 the 1 μM level.
469
470 The test fish were observed immediately after peptide administration to check for signs of
471 distress and any physical or behavioural abnormalities, with the control fish as comparators.
472
473 The fry were counted during Weeks 24, 26 and 33, with a final count during Week 50. The size
474 distribution of the fish populations were established during Week 26 with individuals measured
475 to the nearest millimetre total length and categorized as male, female or juvenile
476 observationally, according to their sexually dimorphic state or lack thereof (juveniles). As
477 between non-reproducing and reproducing female guppies there is no significant difference in
478 body length or protein, only in fat reserves (Wischnath, 1993). Length is a convenient proxy for
479 biomass (i.e. weight), sparing the fish stressful weighing. Weight varies in fish by slightly more
480 than the cube of length (Froese, 2006). In the present study it was not absolute weights that
481 were required but the relative weights of two groups of fish, whose density was assumed to be
482 the same and close to that of the surrounding water, 1g/cm^3. Individual fish lengths were cubed

to provide a pseudovolume, then summed by group to yield a volume ratio between the control and test tanks that did service for 'estimated biomass'.

At Week 33 the fish were moved from their original 30 L tanks to 50 L tanks for continued monitoring. This followed concern about the carrying capacity of the test tank and the potentially suppressive effect of this on fish behaviour.

Receptor

The aim was to determine if any murine brain proteins bound to specimen synthetic peptides, to identify a fecundity receptor. The peptides were the 14mers EPL001 (profecundity in *C. elegans*) and EPL030 (antifecundity) and the 19mer EPL040 (also antifecundity). Biotinylated derivatives of these three peptides were synthesized as part of a biotin-streptavidin purification protocol. Long peptides were selected because biotin was deemed less likely to compromise active sites with those than with the short peptides. The success of the biotinylation process was determined mass spectrometrically. Incubation of the biotinylated peptides with murine brain tissue was followed by streptavidin affinity pulldown of resulting peptide-protein complexes, the bound proteins being identified via liquid chromatography and mass spectrometry.

Biotinylation of peptides

The biotinylation agent biotinamidohexanoyl-6-aminohexanoic acid *N*-hydroxysuccinimide ester (NHS-LC-biotin) was selected (Sigma Aldridge, Poole, Dorset, UK) as the NHS group of this biotinylation agent rapidly and efficiently reacts with free amino groups and NHS-LC-biotin possesses an extra spacer arm rendering the biotin molecule readily available for streptavidin binding (Selo *et al*, 1996). The peptide (1mg) was dissolved in 1mL Reaction buffer (50 mM phosphate buffer (pH 6.5)). 10mM NHS-LC-biotin was added to achieve a 5-

fold molar excess of biotin over peptide in solution, then the reaction mixture incubated at 4°C for 24h. The NHS-LC-biotin was separated from the biotinylated EPL001 using C18 ZipTips ™ (Millipore, Watford, UK) according to the manufacturer's protocol. To determine whether the biotinylation had been successful, HPLC purification of the reaction products was carried out prior to MALDI mass spectrometry. The same process was used for EPL030 and EPL040.

HPLC of biotinylated peptides

The biotinylated peptides were applied to a C18 RP-HPLC analytical column (C18, hypersil GOLD, diameter 250 x 4.6mm, particle size 5p; Thermo Electron, Hemel Hempstead, UK) and eluted with a linear gradient from 25% to 50% of solvent B (100% acetonitrile 0.04% TFA) in solvent A (0.1% TFA) over 25 minutes at a 1mL/min flow rate AT 214nm absorbance detection. NHS-LC-biotin and untreated EPL001, EPL030 and EPL040 were also run under the same conditions.

MALDI TOF mass spectrometry

The biotinylated peptide solution, in the amount of 1µL, was mixed with 1µL of a 10mg/mL solution of α-cyanohydroxycinnamic acid (dissolved in 50/50 water/acetonitrile with 0.1% trifluoroacetic acid added) and allowed to dry on the MALDI plate. The MALDI plate was introduced into a Voyager DE-STR MALDI mass spectrometer (Applied Biosystems, UK) and a full scan mass spectrum was obtained between 50 and 4000 Da, with a laser intensity of 1700, a delay time of 65ns, an acceleration voltage of 20000V and a grid voltage representing 66% of the acceleration voltage.

The spectra indicated that the peptides were successfully biotinylated and also that the biotin bound at up to three positions in all three peptides: Fig. 5 shows the mass spectrum of the EPL001 peptide after the reaction, with a characteristic mass shift of 453Da representing the addition of the biotin group onto the peptide. (The correct mass shift was confirmed by biotinylation of bradykinin, a standard peptide of very similar molecular mass to that of EPL001; data not shown.)

Homogenization of murine brain and incubation with biotinylated peptides

Murine brain tissue, 1g, was homogenized in 9mL Tris-buffer (500pM, pH7.2) at 4^{o}C before the biotinylated peptide was added in 1mL tris-buffer and incubated at 36^{o}C for 1 h. Following the incubation period, further cellular interactions were stopped by transferring each vial to an ice bucket for 30 min.

Separation of proteins bound to biotinylated peptides

Streptavidin magnetic particles, 2.5mL, were added to each 10mL incubation sample to achieve a 10 fold excess of streptavidin over the biotinylated peptide, then incubated at room temperature for 4h. A magnet was placed at the side of the vessel until the streptavidin magnetic particles aggregated, then the top layer decanted off. The streptavidin magnetic particles were then washed with Tris-buffer and the application of the magnet repeated.

Tryptic digestion of streptavidin-protein complexes

Protein-magnetic bead samples were incubated with 15-20ng/ul trypsin in 25mM ammonium bicarbonate (pH 8.4) at 37°C overnight then the sample freeze dried and the protein fragments desalted using C18 ZipTips^{TM} (Millipore, Watford, UK) according to the

manufacturer's protocol.

Protein identification using LC-ESI

The extracted protein fragments were analyzed by nano reverse phase liquid chromatography (Ultimate Pump, Dionex, Netherlands) using an electrospray ion trap MS (LCQ Deca XP, Thermo Electron, Hemel Hempstead, UK). LC-ESI-MS/MS separations were performed using a 10cm x 75mm I.D. pulled-tip capillary column, that was packed in-house with a slurry of 3um, 300A pore size C18 silica bonded stationary phase (PepMap, Dionex, Camberley, UK). The autosampler was fitted with a 5uL injection loop and was refrigerated at 4°C during analysis. After injecting 7.5uL of solution the protein fragments were captured on a C18 trap for 3min and then eluted onto the analytical column, which was washed for 15min with 98% buffer A (0.1% formic acid in water v/v) and protein fragments then eluted using a stepwise gradient of 0% solvent B (0.1% formic acid in acetonitrile v/v) to 65% solvent B in 100min, then to 100% B in 10min with a constant flow rate of 0.2 uL/min. The electrospray MS was operated in a data-dependent mode in which each full MS scan (m/z 475-2000) was followed by three MS/MS scans, in which the three most abundant ions of the protein fragments were dynamically selected for collision-induced dissociation (CID) using a normalized collision energy of 35%. The temperature of the heated capillary and electrospray voltage were 160°C and 1.6kV, respectively. The data were searched against the murine and human databases using Sequest in Bioworks 3.2 (Thermo Electron, Hemel Hempstead, UK) and hits were filtered within Bioworks for amino acid sequence probability (P = 0.001) and for high stringency cross correlation (Xcorr 1+, 2+, 3+ =1.8, 2.5, 3.2).

Molecular modelling

Reproductive peptides were investigated via modelling *in silico*, notably to assess binding sites. Data were produced by Molecular Modelling Proplus, version 6.22, and ChemSite, version 5.10, produced by ChemSW, Accelrys Inc., San Diego, CA92121, USA.

ACKNOWLEDGMENTS:

The nematode studies were conducted by KGD while at Rothamsted Research, Harpenden, Herts, UK, and that institution is thanked for hosting the work. The authors thank Miss C. Royal, formerly of the University of Swansea, UK, and Mrs P. Diffley, who retains that affiliation, for participation in the biotinylation studies and expert technical assistance, respectively. Dave Emery is thanked for logistical support. Alex Cliffe and Alasdair Maltby of ZSL are thanked for caring for and counting the guppies. The authors express their appreciation for personal communications from Jeremy Pearce, John Godliman, Granville Hammond and Mike Bungard.

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Figure Legends

Figure 1. Cumulative larvae produced by 10 nematodes maintained in aqueous buffer and treated with hexamer peptides EPL036 (IEPVFT), EPL037 (LQPAHV) and EPL016 (MKPLTG), compared to untreated controls. (Mean +/– SE ; ANOVA, $p < 0.02$, all test groups versus controls). Each treatment was replicated 4 times.

Figure 2. Fecundity motif: CLUSTAL alignment of proteins orthologous to a protein identified in *C. briggsae* ('CAEBR') as containing IEPVFT (synthesized as the profecundity 6mer peptide EPL036). CAEEL = *C. elegans* 'BRUMA' = *Brugia malayi* (a causative nematode of filariasis).

Figure 3. Guppy fish body lengths at Week 26 (halfway stage): mean length (mm) ± SE of males, females ('Fem') and juveniles ('Fry') in a control tank ('con') and peptide tank ('pep'). The figures in parentheses below the x-axis are the number of individuals in each category. Data include each tank's founding adults (10 male and 10 female). ANOVA p values are given above each pair of bars.

Figure 4. Molecular models of EPL030, a 14mer peptide displaying antifecundity activity in *C. elegans*, and of EPL036, the 6mer profecundity peptide which forms the C terminus of EPL030

Figure 5. Mass spectrum of biotinylated 14mer EPL001.

Table 1. Peptides used. Hexamers in the longer peptides have been underlined. Proline is emboldened in each peptide. (All peptides cited in patents US 8367801 & EP 2234632.)

Designation	Sequence (mer)	Note	Reference
EPL001	MK**P**LTGKVKEFNNI (14)	Inhibits compensatory renal growth in rat	Haylor *et al*, 2009
EPL030	KLKMNGKNIE**P**VFT (14)	Anagrammatical control I	Davies & Hart, 2008
EPL040	KLKNMGNKIE**P**VFTGSGSK (19)	Anagrammatical control II, with linker	Davies & Hart, 2008
EPL036	IE**P**VFT (6)	Common motif between EPL030 and EPL040 with bioinformatic Blast hit to *Caenorhabditis briggsae*	This report
EPL037	LQ**P**AHV (6)	*C. elegans* ortholog to EPL036	This report
EPL016	MK**P**LTG (6)	Motif containing proline from EPL001	This report

Table 2. Worm survivorship of a starting population of 10 individuals maintained in aqueous buffer; each treatment was replicated 4 times. Controls were unexposed to hexamer peptides. Data are in days; ns = non-significant in comparison with controls; ANOVA * = $p < 0.05$; ** = $p < 0.005$

	Controls	EPL016 (MKPLTG)	EPL036 (IEPVFT)	EPL037 (LQPAHV)
Average lifespan	5.4	5.0 (ns)	5.1 (ns)	6.0 (ns)
50 % dead	4.0	4.6 (ns)	4.9 (*)	6.0 (**)
100 % dead	10.3	8.8 (ns)	7.3 (ns)	10.3 (ns)

Table 3. Cumulative descendants of two founding populations of guppies, each comprising 10 males and 10 females. Controls were untreated. Test guppies were exposed to the 6mer peptide EPL036 via their tank water. 'Week 0' = immediately prior to study commencement.

Week	Control	Peptide	Peptide as % Control
0	0	0	0
24	23	68	295
26	25	103	412
33	86	142	165
50	202	177	88

753

754 Table 4. Receptor hunt: identification of candidate receptors in murine brain to 3 biotinylated
755 synthetic peptides (the 14mers EPL001 and EPL030 and the 19mer EPL040), purified using
756 streptavidin and analysed via mass spectrometry (MS).

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Synthetic peptide used	Amino acid sequence from MS	Protein database hit	Function/ location	MH^+	z	X_{corr}	Ions
EPL001	KEGDGSATTDAAPATSPKA	Growth Associated Protein 43	Substrate of protein kinase C, located in developing neuronal cells	1575.7	2	2.68	13/32
	RSDGKISEQSDAKL	Mitochondrial ATP Synthase (α-subunit)	Involved in energy production, membrane bound	1264.6	2	2.67	18/22
	RGIHGEHSESEGGVYDISNKA ——————— RLGLTEFEAVKQM	Arginine Kinase	Maintenance of ATP levels, rapid energy production	2016.0 ——— 1235.4	3 — 2	4.71 ——— 2.04	31/72 ——— 15/20
EPL030	RRFQEDIEITNVFNVYAPPKDFY	Nuclear Hormone Receptor	Involved in embryonic development and egg laying	2543.8	2	2.68	22/80
	RYLAEVASGDDRNSVVEKS	14-3-3 Family Member	Thought to regulate lifespan	1852.9	2	3.14	16/34
EPL040	RGIHGEHSESEGGVYDISNKA	Arginine Kinase	Maintenance of ATP levels, rapid energy production	2016.0	3	4.12	28/72

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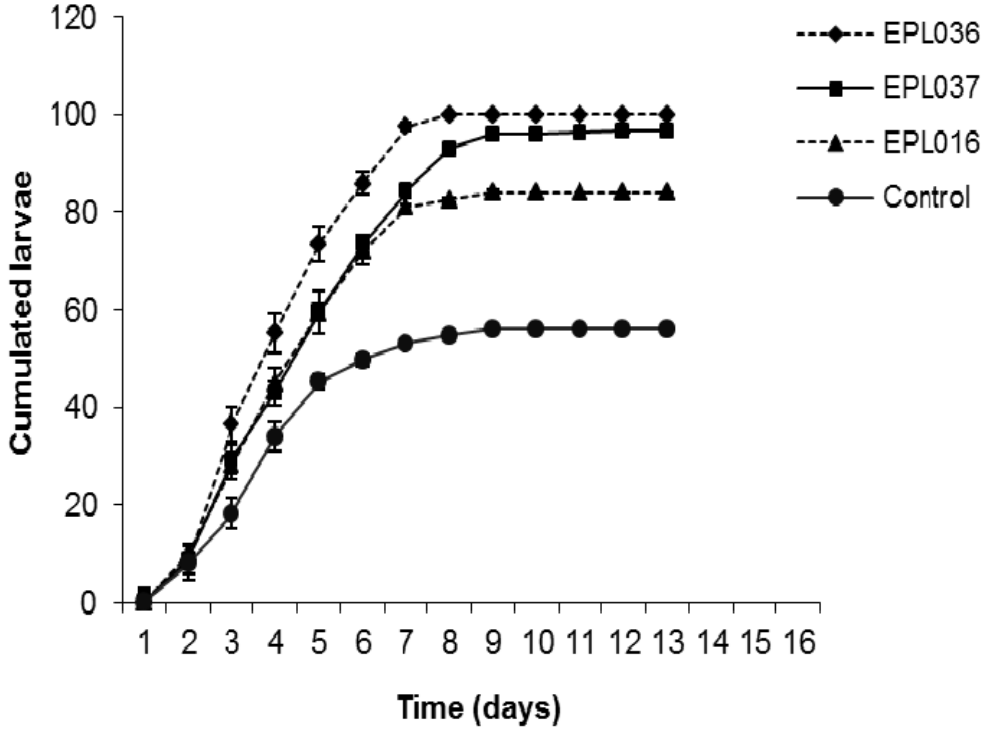

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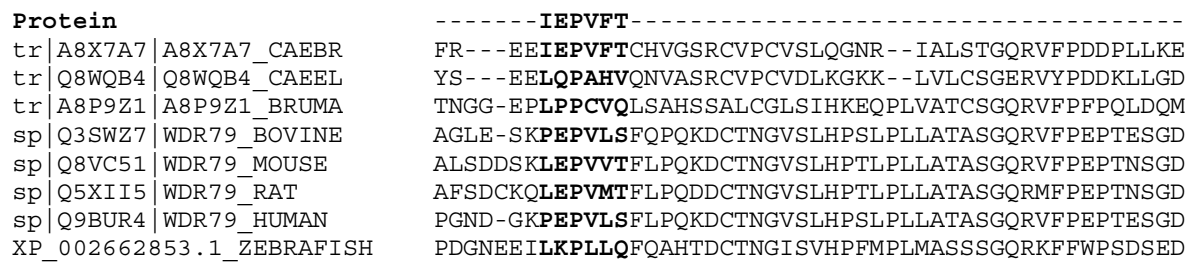

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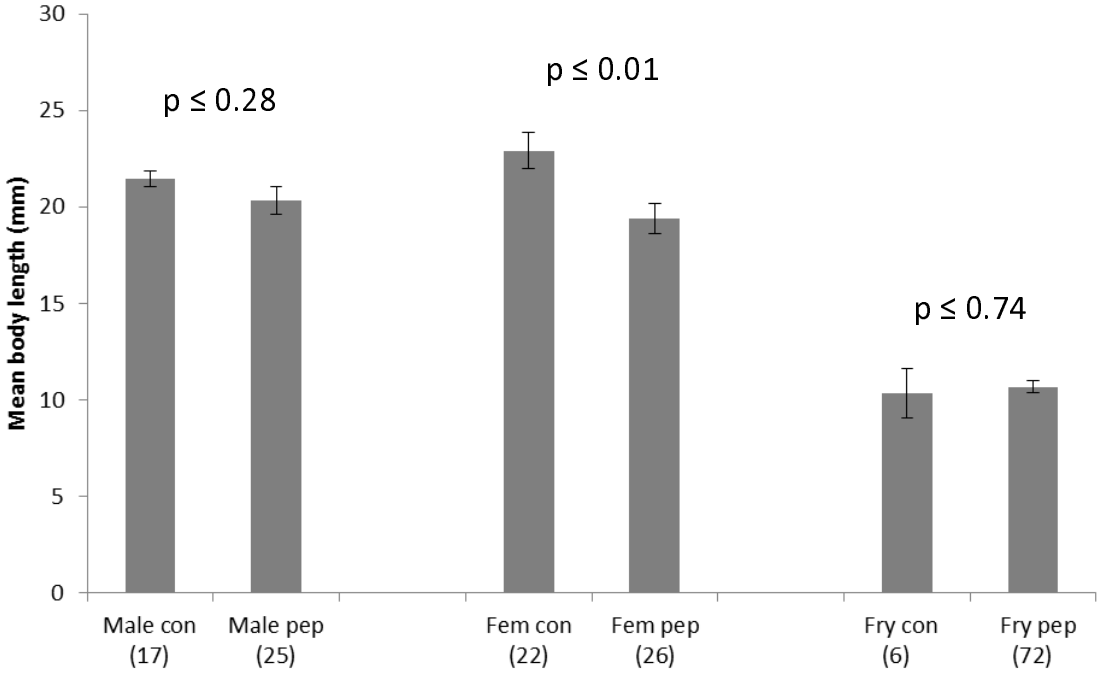

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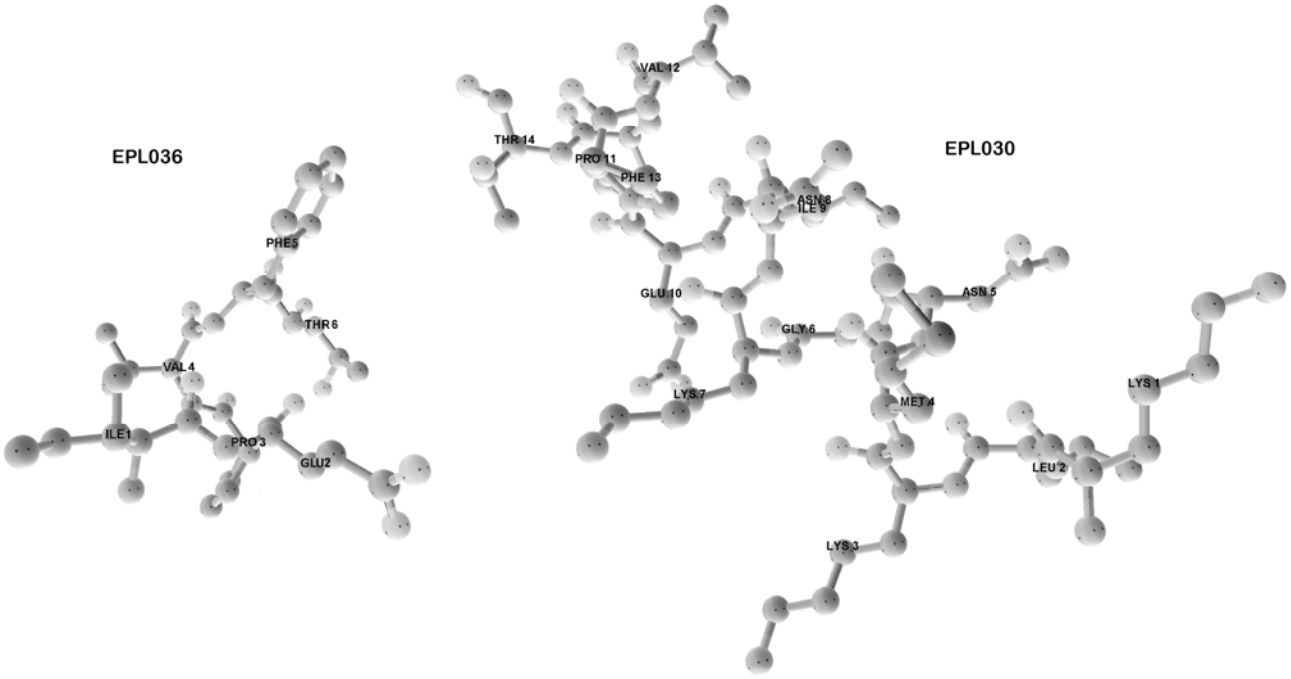

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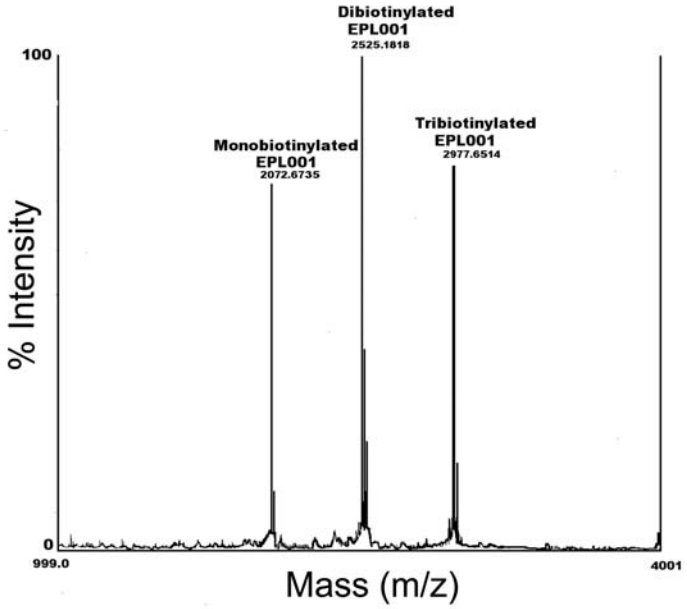

Figure 5. Mass spectrum of biotinylated 14mer EPL001